

## COMMENTARY

# Mayfly metamorphosis: Adult winged insects that molt

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Mayflies (order Ephemeroptera), insects favored as food by freshwater fish and as models for the artificial lures of fly fishers, live double lives. During their immature (nymphal) stages, they pursue an underwater career for 1 y or 2 y, molting their cuticular exoskeleton on numerous occasions as they grow. As in other insects, their future wings grow only slowly, but, as maturity approaches, the organs of flight grow rapidly, and the adult insect emerges from the water in a completely different form than the nymph. All winged hexapods (Pterygota) undergo a metamorphosis of more or less this kind, but mayflies are unique among living insects in having more than one winged developmental stage (1), the final imago or adult instar being preceded by a short-lived subimago (called by anglers a “dun”) (Fig. 1). A paper in PNAS by Kamsi et al. (2) now presents a molecular analysis of the hormonal and cellular control of metamorphosis in a model ephemeropteran species, *Cloeon dipterum*, casting fresh light on the evolutionary significance of the mayfly subimaginal stage.

It has long been known that the mayfly's two different winged instars are distinct developmental stages, separated by a highly compressed but otherwise normal molting cycle (3). In this, mayflies appear intermediate between all other winged insects, which cease to shed their skins on achieving the adult condition, and wingless insects from the orders Archeognatha (jumping bristletails) and Zygentoma (silverfish and firebrats), which undertake multiple molts as adults while they continue to reproduce (4).

The paper by Kamsi et al. (2) unequivocally shows that the molt of the last nymphal stage of *C. dipterum* to the subimago makes use of the same hormones, receptors, and cellular signals of the MEKRE93 pathway (5) that regulate metamorphosis in all other insects. Specifically, the two successive adult molts of *C. dipterum* are triggered by successive pulses of the molt-initiating hormone ecdysone, a steroid secreted by the prothoracic glands (PG); the first pulse is at the end of the final nymphal instar, while the next occurs immediately on

molting to subimago. As in other insects, whether the molt is a metamorphic one is regulated by the sesquiterpenoid juvenile hormone, JH, the action of which is mediated by expression of Krüppel homolog 1 (Kr-h1), a high-level transcription factor. In *C. dipterum*, Kr-h1 is strongly suppressed from the start of the final nymphal stage. The expression of the adult “master gene” *E93*, another high-level transcription factor associated in all other insects exclusively with the adult condition, is concomitantly increased from day 1 of the final nymphal stage, thus committing the developing next instar to an adultiform condition. Further, application prior to the initiation of the nymphal–subimaginal molt of the JH mimic, methoprene, results in increased expression of Kr-h1 and failure to express *E93*, thus preventing metamorphosis and causing the formation of a supernumerary nymph, an outcome consistent with the status quo role of JH in specifying continuation of the nymphal condition, exactly as in all other hemimetabolous insects (6–8). The involvement of these now well-understood



**Fig. 1.** Subimago of the mayfly *C. dipterum*. Image credit: Isabel Almudí (Centro Andaluz de Biología del Desarrollo, Seville, Spain).

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cellular control pathways in specifying the developmental events of metamorphosis therefore unambiguously identifies the mayfly subimago as developmentally similar to the adult stage of other insects, and quite unlike a modified larval or pupal stage. This is consistent with previous work that found the subimaginal transcriptome of another mayfly, *Cloeon viridulum*, to be most similar to that of the adult (9).

But why, of all insects, do only mayflies molt again as adults? It is significant that nymphs of all the more than 3,000 extant ephemeropteran species occupy exclusively aquatic niches. One long-standing hypothesis is that mayfly nymphs are so highly adapted to living underwater that the transition to the aerial adult form is too great to be accomplished in a single molt. In this view, the subimago is not really an adult at all but a highly modified nymphal stage, functionally equivalent to the pupal stage of those holometabolous insects that undergo a “complete” metamorphosis (10). While certain developmental aspects of the subimago are, indeed, morphologically transitional in this way (e.g., the increasing length of the legs and caudal cerci), Kamsi et al. (2) have now convincingly refuted the idea that the subimago is a modified nymphal stage; it is clear that the mayfly subimago is a proper adult.

The adaptive benefit of the subimago is perhaps better explained by the mayfly’s abrupt transition from an aquatic to an aerial life. Unlike the aquatic nymphs of dragonflies and damselflies, or those of less closely related stoneflies, most mayflies do not crawl onto land in order to become adults; instead, they molt at the surface of water and take flight almost immediately (1). Once extricated from the exuvia, the subimago’s wings are almost immediately functional (i.e., the insect can fly) but differ from those of the adult in that they are covered with a down-like covering of tiny hairs (microtrichia) and possess a fringe of trailing hairs (Fig. 1). These features have been suggested to be hydrofuge adaptations that allow rapid shedding of water and avoid wetting; emergence from the water during metamorphosis is a time of great risk for mayflies, and entrapment by adhesion at wet surfaces is a serious hazard (1). The hairy wings of the subimago reduce this risk. Once emergence has taken place, the subimago’s job is done, and the insect molts to the adult stage, in which the wings are smooth and thin.

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So, is it the subimago or the adult that is the evolutionary innovation? Belles (11) has pointed out that adult molting would be maladaptive for most insects, and that a key evolutionary change in basal winged insects must have been the degeneration of the adult’s PG, an event that terminates further secretion of ecdysone, preventing the initiation of further molting cycles. Extracting new wings from inside a previous set of functional flattened wing vanes would be mechanically difficult and risks getting lethally stuck. Moreover, to be able to molt again, the wings have to retain epidermal cells between layers of cuticle, and, during molting, have to carry additional water in the form of molting fluid present between successive adult cuticles, both of which would increase wing weight and seriously constrain flight ability. These

considerations explain why insects other than mayflies do not molt as adults. As we have seen, however, it may nevertheless be advantageous to mayflies to be able to molt once again as an adult; this would require destruction of the PG to be delayed.

Such reasoning is consistent with the finding that expression of the adult-specifying transcription factor E93 in the absence of Kr-h1 causes degeneration of the PG. Using RNA interference to prevent the expression of E93 in the cockroach *Blatta germanica*, another hemimetabolous insect (which, of course, does not normally molt as an adult), prevents cell death in the PG and allows initiation of a second adult molt (12). Downstream of E93 are other gene products, including the negatively regulated Inhibitor of Apoptosis-1 (13). We can therefore predict that, when a mayfly nymph molts to the subimago, diminished E93 expression in the PG should protect the glands from degenerating. Accordingly, Kamsi et al. (2) find that, unlike the situation in the cockroach, in the final nymphal stage of *C. dipterum*, E93 is expressed at only a low level (much less than seen in the adult). Perhaps this lower E93 titer is enough to promote the formation of new adultiform cuticular structures in the subimago but is too low to cause PG degeneration. Alternatively, it may be that the level of E93 messenger RNA appears low in the mayfly because E93 is expressed only in a subset of tissues. If E93 were absent in the PG, then we might expect that degeneration of the gland would not occur. Either way, PG survival would permit another round of molting.

Are mayflies really an evolutionary intervening stage between apterygotes and other winged insects, or is their additional adult molt a secondarily derived character? Kamsi et al. (2) suggest that adult molting may have been widespread long ago among the first winged hexapods. It is true that we can be confident that all present-day insects are indeed descended from wingless ancestors that continued to molt as adults just as modern apterygotes do. Unfortunately, however, the relatedness of Ephemeroptera to other winged insects is uncertain. It is undisputed that mayflies are ancient, but neither the fossil record nor molecular phylogenomics gives strong support to the idea that mayflies were the first pterygotes or even ancestral to other orders. The earliest fossils of winged hexapods are not mayflies but Paleodictyoptera, an extinct order of insects that give no hint of a mayfly-like aquatic lifestyle (14, 15). While it has been asserted (e.g., ref. 16) that some fossil insect wings from this and other extinct orders are derived from subimaginal-like stages, the criteria for identifying them as such are unclear. The earliest date for a fossil mayfly is 240.5 Ma, some 82 Ma younger than the earliest known insect (15).

Among extant insect orders, Ephemeroptera are conventionally grouped together only with Odonata (dragonflies and damselflies) within the division Paleoptera, comprising those insects that share the trait of being unable to fold their wings when not in use. All other living insects have sophisticated wing-folding mechanisms and, together, form the division Neoptera. Although it is logical to suppose that insects with folding wings must have evolved from those with less complex wing articulations, this does not prove that Neoptera are derived from Paleoptera, a grouping that is not well supported by molecular data. The widely accepted consensus insect family tree based on transcriptomic sequencing (17, 18) shows that, although Odonata and Ephemeroptera are most closely related to each other, they are deeply separated, with an inferred date of divergence of >350 Ma, and both are even more distantly related to all other insect orders. Crucially, although Neoptera is indeed recovered as a clade that includes all extant nonpaleopteran insects, it is not possible to place either

of the orders, Ephemeroptera or Odonata, as ancestral to the other, and both evidently diverged from Neoptera at an early date (19).

While it is possible that, during early insect evolution, there was a gradual reduction of the number of adult instars from many to just two, as represented by mayflies, and that later insects went on to reduce adult molting still further, an alternative scenario is possible. In this, Ephemeroptera were not ancestral to other insect orders but were secondarily derived from a preexisting winged ancestor, which had already reduced the number of adult stages to one through the destruction at metamorphosis of its molt-initiating endocrine glands. This early loss of adult molting would

have been driven by selection against the hazardous ecdysis of fully formed adult wings. The subimago would then be an innovation of an insect that occupied an aquatic nymphal niche, was derived from an original fully adult stage, and was selected because the acquisition of special adaptations of its wings allowed a quick, safe exit from the water at the time of emergence. This came, however, at a cost, as the same traits would have diminished the insect's aerial agility. This potential cost to fitness could, however, be sidestepped through decoupling selection on wing function by delaying degeneration of the PG and other tissues, thereby allowing the formation of an additional, second adult stage with lighter, more aerobatic wings.

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